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TITLE OF THE INVENTION (500 characters max)					
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Methods for signal amplification used in analyte detection

This invention relates to a method for improving the sensitivity of analyte detection using a signal amplifying system (SAS) having a structure consisting of multiple signal groups such as chemiluminescent compounds, a carrier entity or entities and one or more affinity groups specific for an analyte.

This invention is a signal amplification system (SAS) comprising three functional components. The invention is better understood by referring to Figure 1. In general, the SAS comprises a large number of signal groups such as chemiluminescent compounds or their derivatives (such as their precursors) 1, which are attached to a carrier entity 2, which also carries one or more affinity groups 3 specific for an analyte or analytes. One SAS unit can have multiple copies of chemiluminescent groups and one or more copies of affinity groups. Preferably the number of the signal groups in each SAS unit should be same or similar for high sensitivity detection. In order to detect certain analyte, the affinity group in SAS need to have specific affinity for the analyte.

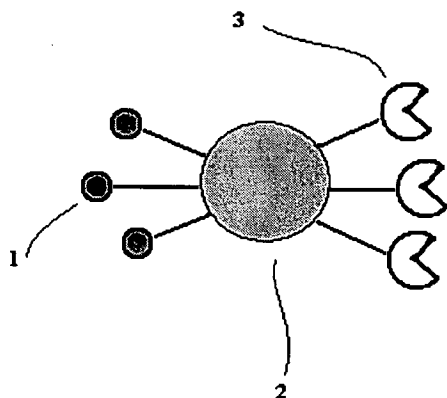


Figure 1 signal amplification system (SAS)

Figure 2A depicts one specific embodiment of the invention, where the affinity group 3 is attached to the chemiluminescent compounds or their derivatives 1, which are coupled to the carrier 2.

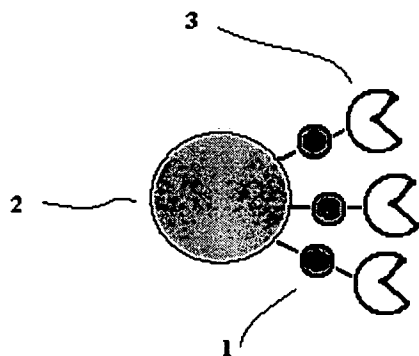


Figure 2A Schematic view of one specific embodiment of the invention, the affinity group 3 is attached to chemiluminescent compounds 1

Conversely, as depicted in Figure 2B, the chemiluminescent compounds or their derivatives are attached to carrier 2 through the affinity groups 3. The key aspect of these embodiments is that either the affinity groups or the chemiluminescent compounds, but not both, are directly linked to the carrier.

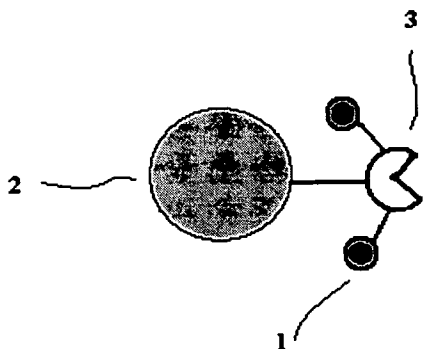


Figure 2B Schematic view of another specific embodiment of the invention, chemiluminescent compounds 1 is attached to the affinity group 3

Figure 3 illustrates a preferred embodiment of the invention, where the chemiluminescent compounds or their derivatives 1 are encapsulated in microparticles or the like 2 and the affinity groups 3 are conjugated on the surface of the particles. This method allows encapsulation of large number of signaling molecules while large numbers of affinity groups can still be labeled to particle surface.

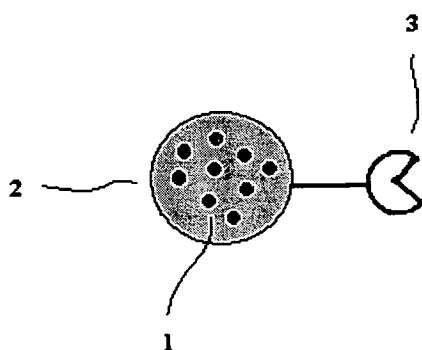


Figure 3 Schematic view of a preferred embodiment of the invention, where the chemiluminescent compounds or their derivatives are encapsulated in microparticles

Figure 4 depicts another preferred embodiment of the invention, where the affinity groups 3 or chemiluminescent compounds 1 or both are coupled to an intermediate carrier 4 (the second carrier), which is subsequently coupled to the first carrier 2. This method allows labeling of more signaling molecules. This scheme of coupling can be repeated a number of times to allow the labeling of

even more signaling molecules. When more than one carriers are used, it is preferred that the affinity groups are only coupled to the outer most layer of carrier. It is understood that carriers may be the same type or different type in composition. For example, both first carrier 2 and second carrier 4 can be microparticles. Alternatively, the first carrier 2 may be microparticles whereas the second carrier 2 may be a polymer, e.g., polylysine.

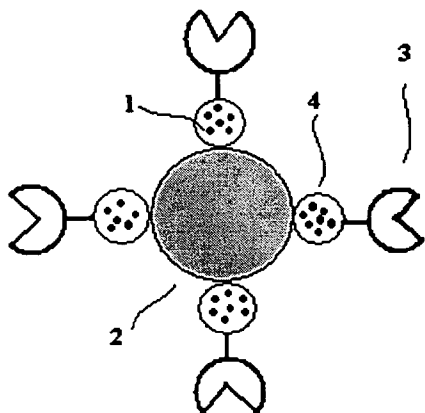


Figure 4 depicts another preferred embodiment of the invention, where the affinity groups or chemiluminescent compounds or both are coupled to an intermediate carrier (the second carrier), which is subsequently coupled to the first carrier.

Detection of an analyte is normally accomplished via specific binding of SAS units to a solid support such as microwell plate or magnetic particles. After washing away unbound SAS, the bound SAS can be measured immediately for chemiluminescence or after the chemiluminescent compounds are released from the carrier. Chemiluminescence can be detected using a luminometer or electro-luminometer. The magnitude of amplification is related to the number of chemiluminescent groups or molecules on an SAS unit. The more chemiluminescent groups or molecules on a carrier entity, the higher the amplification magnitude can be.

Appropriate chemiluminescence here includes, but is not limited to, both direct chemiluminescence such as that generated with enzymes or acridinium and electro chemiluminescence such as that generated with rare earth elements. Chemiluminescent compound or compounds can be anything that generates light signal under appropriate conditions or the precursors that gives rise to such compounds. Examples for this type of compounds include both chemiluminescent compound (e.g., acridinium, proteins that can generate light, enzymes that can catalyze chemiluminescence reaction) and electrochemoluminescent agents (e.g., certain organic compounds or metal elements in appropriate chelators). It also includes those compounds that function in a fashion similar to a chemiluminescent compound. For example, rare earth elements such as europium emit long half-life fluorescence, which permits time-delayed measurement of the fluorescence after withdrawal of excitation light. Because the fluorescence is measured in the absence of excitation light, this type of compounds functions in a fashion similar to a chemiluminescent compound.

Chemiluminescent compounds may be coupled to the carrier either permanently (non-releasable) or through a cleavable (releasable) bond, e.g., photo-labile bond, chemical –labile bond such as an acid sensitive bond or a detachable bond, e.g., polynucleotide base pairing. Release of chemiluminescent compounds from the carrier before detection may improve detection efficiency.

The affinity group or groups can be any chemical or biological functionality with specific affinity for certain analytes. They include, but are not limited to, polynucleotides, antibody, antigen, nucleic acid binding species (such as aptamers, which is nucleic acid sequence that can bind with non nucleic acid target), chelator and etc. The affinity group may be indirectly coupled to the carrier through a linker or an adaptor through, for example, a ligand-receptor binding (e.g., biotin-avidin) or hybridization between a polynucleotide and its complementary sequence.

The carrier entity can be a polymer, a microparticle, or a combination of the two. Appropriate natural or synthetic polymers include, but are not limited to, oligomers (such as peptides), linear or cross-linked polymers (such as poly lysine, poly acrylic acid, proteins) or highly branched macromolecules (such as dendrimers). A chemical, biological or physical entity can be used as a carrier as long as it has multiple functional groups that allow direct or indirect conjugation of multiple numbers of chemiluminescent compounds and affinity groups. The more functional groups a carrier has, the better amplification it will provide. A preferred carrier is a microparticle because it can be coated with a large number of functional groups such as carboxyl group or primary amine. Preferred size of microparticles is in the range of nanometer to micrometer in diameter. Suitable microparticles include, but are not limited to, microspheres, nanoparticles, liposomes, microcapsules and etc.

A carrier may be first directly or indirectly coupled with chemiluminescent compounds or affinity groups or both and then directly or indirectly conjugated to yet another carrier of the same type or different type. For example, acridinium and an oligonucleotide probe can be first coupled to polylysine to generate an acridinium-polylysine-oligonucleotide probe complex, which is subsequently conjugated to a microparticle. This carrier-to-carrier coupling reaction can be repeated a number of times to achieve further amplification prior to, during, or after contacting the analyte.

When microparticles or the like are used as carriers, chemiluminescent compounds may be encapsulated in the particles. Encapsulation may be performed through physical means, e.g., trapping, internal adsorption, or through chemical means, e.g., covalent coupling. Alternatively, chemiluminescent compounds can first be directly or indirectly coupled to a carrier (e.g., a polymer or nanoparticles) and then encapsulated in the particle. When chemiluminescent compounds are encapsulated in particles, it is preferred that the particles are dissolved, swelled, or perforated prior to chemiluminescence detection so that the chemiluminescent compounds are in contact with reagents or under conditions that trigger chemiluminescence reaction. One could use certain physical means or certain chemicals (such as organic solvent, strong acid or base, preferably be

heated) to swell or partially or completely dissolve or destroy the microparticle, which releases the trapped chemiluminescent groups and may improve detection efficiency. For example, polymer microsphere made from monomers containing high concentration of 4-amino styrene or acrylic acid can be dissolved with base or acid respectively, similar to the method used for controlled release in pharmaceuticals. The chemiluminescent compounds encapsulated could be in the form of aggregate, e.g., small particles, powder, or crystals, which are preferably in nano meter size range. For example, when rare-earth element such as Eu is used, it could be in the form of Eu metal particles, Eu oxide particles or other Eu containing compounds aggregate. The SAS particles containing these forms of Eu or other rare earth elements are also useful for fluorescent detection or electro-chemiluminescent detection. However, the encapsulated rare elements are preferably released from the particles using physical means or certain chemicals (e.g., organic solvent, strong acid or base). Suitable chemicals for encapsulating chemiluminescent or fluorescent compounds include, but are not limited to, polymers such as polystyrene and small organic compounds such as Si containing compounds can also be used to coat chemiluminescent or fluorescent compound particle to give the encapsulated SAS microparticles. For example, 3-aminopropyltrimethoxysilane APTMS can be used to coat Eu_2O_3 nano particle or the like. The coated Eu_2O_3 particles or the like can be conjugated with affinity groups and used as an SAS for the assay. It is preferred that the particles be treated with certain chemicals (e.g., acid, organic solvent in acidic condition) to dissolve the particles and release Eu into a soluble form prior to fluorescence detection preferably in the presence of a fluorescence-enhancing agent such as beta-diketone. The released Eu or the like can also be used for electro-chemiluminescent detection.

Rare earth elements or the like may be encapsulated in microparticles in the form of ions through a chelator. The Eu chelate, e.g. EDTA-Eu, beta-diketone-Eu, TTA-Eu-TOPO can be incorporated into the monomer covalently. For example appropriate chelator including, but are not limited to, isothiocyanatobenzyl-EDTA or TTA derivatives having an acid group or EDTA-5-aminosalicylic acid or 4,7-bis(chlorosulfonyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) can be coupled to amine containing monomers such as 4-amino styrene through an amide bond linkage. The resultant Eu chelate containing monomer can be subsequently polymerized into microparticle or copolymerized with other monomer to give desired microparticle. Alternatively, the chelator, e.g., diketone, can be first incorporated into the monomer, which is polymerized or copolymerized with other monomer to form desired microparticles. The microparticles are then swelled or perforated and then incubated with Eu salt solution to allow Eu incorporation via the chelator molecules. The diketone-Eu containing microparticle allows high sensitive fluorescent detection even without releasing the Eu inside the particles.

The more signaling molecules are there in a SAS microparticle, the better detection sensitivity can be achieved. Since larger sizes of microparticles can normally carry more signaling molecules, larger SAS microparticles will result in better detection sensitivity. Preferably, the SAS microparticles (such as a polystyrene microspheres) are larger than 100 nanometers in diameter. More preferably, the SAS microparticles are at least 500 nanometers in diameter. Most

preferably, the SAS microparticles are at least 1000 nanometers in diameter. In our study, the SAS microparticles of 2~3 micrometers in diameter give satisfactory detection sensitivity. It is preferable that millions or even higher amount of signal molecules to be coupled or entrapped to these microparticle for very high amplification effect.

It is within the scope of this invention that fluorescent compounds or other signaling compounds are used instead of chemiluminescent compounds as the signaling molecules in the SAS, and therefore the detection is fluorescence instead of chemiluminescence if fluorescent signal molecule is used.

When used for analyte detection, the SAS can be used in a “sandwich” format or its variation. Generally, an analyte is first immobilized onto a solid phase using an affinity group such as an antibody, which preferably binds to a different epitope than the affinity group on the SAS. After wash away unbound entities, the SAS is added to a binding solution that permits the binding of SAS to immobilized analytes. After washing away unbound SAS, the bound SAS is detected for associated chemoluminescent compounds using an appropriate instrument such as a luminometer. The mixing and binding of analyte to solid phase and SAS can also be performed in one step simultaneously.

Preferably, the SAS or the signal molecules such as chemiluminescent or fluorescent molecule it carries is separated from the solid phase capture surface (such as micro plate well or magnetic beads) before detection, which may reduce potential interference from the capture surface. The capture surface itself can produce significant background fluorescence and the particles can block light signal. Physical means (e.g., heat) or chemical means (e.g., appropriate acid or base, protein denaturing reagents such as guanidine isothiocyanate) or the like could be used to disassociate the sandwich structure or to release the chemiluminescent molecules from the SAS, thereby separating the signal molecules from the capture surface. If the SAS particle dissolution step is involved during the assay, the magnetic capture particles, if used, can be made resistant to the dissolution condition by using, for example, magnetic beads made of highly cross-linked polymer, which allows the separation of magnetic particles from SAS or signaling molecules. In traditional microwell plate based assays, the capture surface is normally coated with affinity groups, e.g., antigens or oligonucleotides, which often generate background fluorescence. In this situation, the dissociated SAS or signal molecules or fluorescent probes are preferably transferred to a clean well for detection.

If the chemiluminescent group is organic chemiluminescent compound, a luminometer can be used for detection; if the signal group is electro-chemiluminescent agent such as rare earth element, an electro-chemiluminescent detector can be used for detection. One example of SAS for electro-chemiluminescent detection is given here: multiple Eu chelates-single or multiple dendrimer or linear polymer (such as poly amino styrene)- antibody (“-” generally refers to a linker or a bond). The carrier could also be a microparticle instead of a polymer. If the chemiluminescent compounds are coated on the surface of the particles, it is not necessary, though preferred, to release the chemical to the solution prior to detection.

Further more the SAS can also be used in combination with other instruments. For example, it could be used in combination with a wave guide sensor like device to further increase its detection limit.

EXAMPLE 1

Figure 5 below illustrates an example using SAS technology. The assay is aimed to detect certain antigen 5 in the sample containing other molecules 4. Antibody specific for antigen 5 is immobilized on the surface of a solid phase support such as a microwell plate well 1. A microsphere-based SAS 3 having multiple chemiluminescent molecules 2 and antibody specific for antigen 5 is added to the well with the sample. Upon binding, SAS will be immobilized on the well wall. After washing to remove the unbound SAS and analyte 4, the chemiluminescent molecules 2 is released from the SAS microsphere using chemicals or light that can cleave the bonds between chemiluminescent molecules 2 and the microsphere. The released chemiluminescent molecules 2 can be readily detected using a luminometer or electro-chemiluminescence detector. Although releasing is preferred, the bound chemiluminescent compounds can also be detected without being cleaved. The chemiluminescence intensity is proportional to the amount of antigen 5 in the sample.

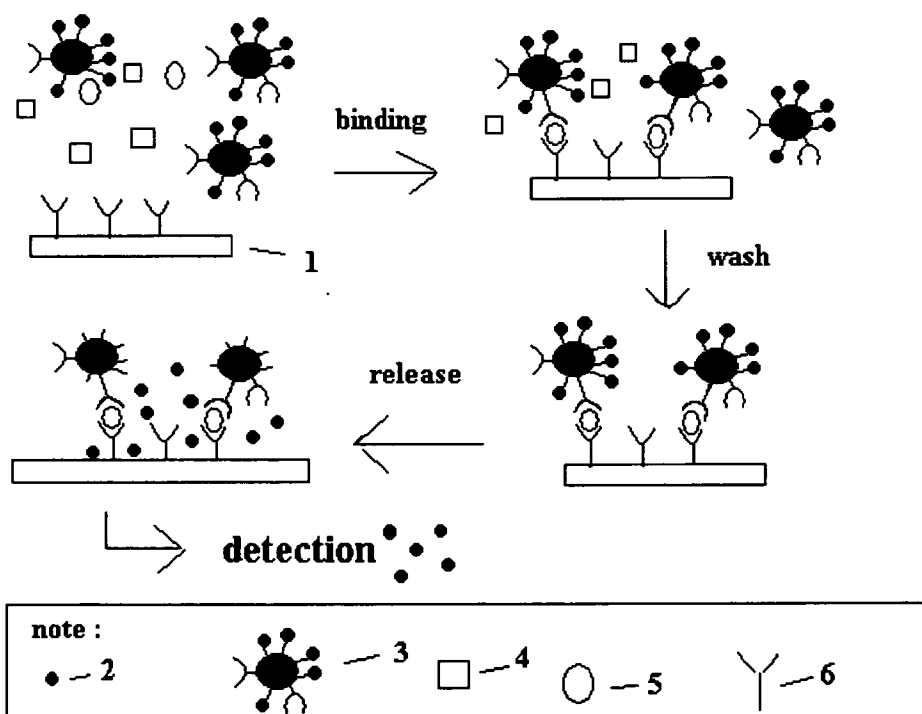


Figure 5 An example for analyte detection using the SAS

EXAMPLE 2

Figure 6 illustrates yet another example, which uses magnetic particles as solid phase support and an SAS with chemiluminescent molecules encapsulated in microparticles. In this case, both the magnetic particles 9 and SAS particles 8 are coated with distinct affinity groups, e.g., polynucleotide probes 6 and 10 that

hybridize with different regions of HIV-1 viral RNA for the detection of this virus. The magnetic particles are preferably approximately 3 micrometer in diameter and are coated with functional groups such as carboxyl group, which facilitates the labeling of affinity groups such as oligonucleotide probe. An example for suitable magnetic particles is Dynabeads M-270 coated with carboxylic acid (available from Dynal Biotech, Oslo, Norway).

An assay requires at least one set of probes, e.g., polynucleotide probes 6 and 10, although more than one set of probes is preferred since more probes may provide stronger binding. The probes may not necessarily need to be conjugated to magnetic particles or SAS unit if the probes also contain a ligand, e.g., biotin, or a specific nucleic acid sequence, which can be used for binding to magnetic particles or SAS units through ligand-receptor binding or nucleic acid hybridization.

The sample to be tested is first treated with appropriate reagents and conditions, e.g., a buffer containing guanidine thiocyanate, to denature the proteins and release the nucleic acids in the sample. Magnetic particles 9 are added and incubated for an appropriate period time at appropriate temperature. The capture magnetic particles 9 are then washed several times to remove unbound entities 4, suspended in a solution that promotes specific hybridization, and then incubated with SAS microparticles 8. If HIV-1 RNA 5 is present, the magnetic particles and SAS microparticles will be bound together through HIV-1 RNA. After washing away unbound SAS microparticles using a magnet or its equivalent, the bound SAS microparticles are dissolved with an appropriate solvent, e.g., dimethylsulfoxide for polystyrene particles, and released chemiluminescent compounds 2 are detected with an instrument such as luminometer.

Because only a few analyte molecules are needed to provide stable binding between magnetic particles and SAS particles and because each SAS microparticle is encapsulated with a large number of chemiluminescent molecules, the signal is greatly amplified. The sensitivity of the assay depends on several factors, including the minimal number of SAS microparticles that can be detected and the efficiency of removing unbound SAS microparticles (the background). For example, if the minimal number of SAS microparticles that can be detected is ten (10) and all unbound SAS particles are removed, then the sensitivity of the assay is ten HIV-1 RNA copies. When magnetic particles and polystyrene microparticle based SAS labeled with acridinium as chemiluminescent molecule are used for detecting HIV-1 viral RNA, as low as ten copies of virus can be easily detected. To increase the stability of the capture magnetic particles-analyte (here is HIV-1 RNA)-SAS complex, it is preferred that more than one type of polynucleotide ligands (probes) targeting different regions of the analyte (HIV-1 RNA) are coated onto SAS or onto both capture surface (here is magnetic particle) and SAS unit. The use of multiple different ligands on each magnetic particle and SAS unit can improve the sensitivity of HIV-1 RNA detection. In this case even one copy of HIV-1 RNA could result in multiple polynucleotide binding pairs between the analyte and capture surface and between the analyte and the SAS. Alternatively, multiple different strands of binding DNA sequence targeting different regions of the HIV RNA could be coupled to different unit of the magnetic beads and the SAS carrier, e.g. each set of magnetic bead and the SAS

have their own type of binding sequence. In this case, the HIV RNA may be cleaved into small fragments before binding. Higher amount and higher concentration of the affinity groups on the capture surface (such as the micro plate well or magnetic beads) and on the SAS can also result in more stable sandwich structure due to the multiple valent ligands effect for increased detection sensitivity.

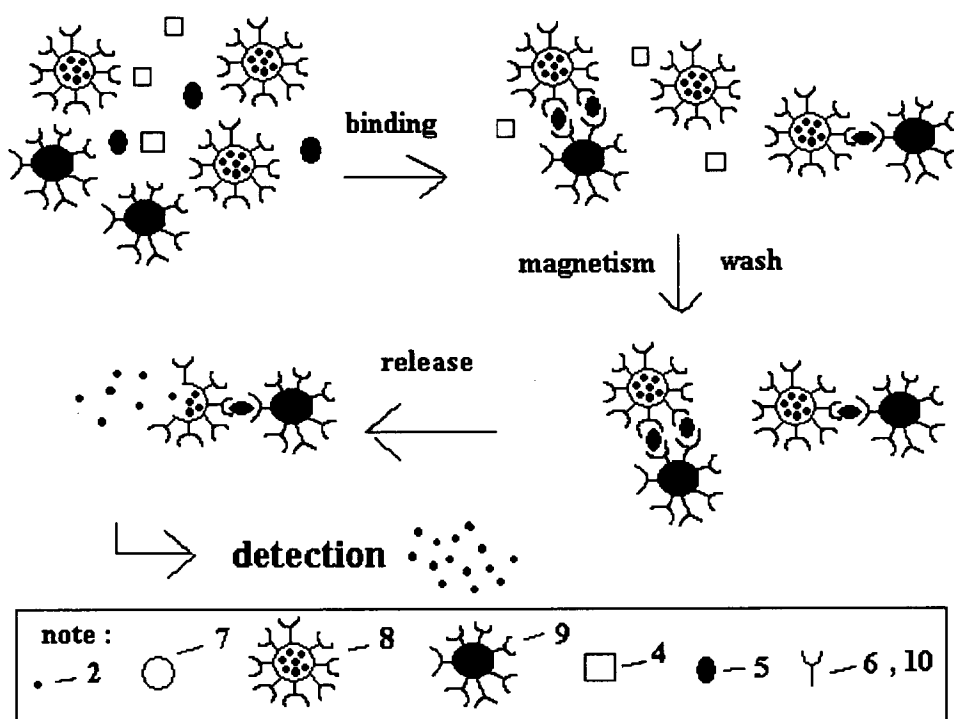


Figure 6: Another example of analyte detection with the SAS technology: the use of magnetic particles as solid phase support.

EXAMPLE 3

This example shows how the SAS technology can be used for sensitive detection of a particular species or class of species of bacteria using a nucleic acid target, e.g., tRNA, ribosomal RNA. Similar to the HIV-1 assay, there needs to be at least one pair of probes. Here in this example, the probes are relatively long oligonucleotides that contains two hybridization domains, one of which is specific for the target nucleic acids whereas the other domain is specific for the oligonucleotides conjugated on magnetic particles for one of the probes or for the oligonucleotides conjugated on SAS units for another probe. In this example, we use Probes A and B, which contain hybridization domains for magnetic particles and SAS, respectively. The magnetic particles are preferably approximately 3 micrometer in diameter and are coated with functional groups such as carboxyl group, which facilitates the labeling of affinity groups such as oligonucleotide probe. An example for suitable magnetic particles is Dynabeads M-270 coated with carboxylic acid (available from Dynal Biotech, Oslo, Norway).

Acridinium and oligonucleotide probe can be first coupled to polylysine to generate an acridinium-polylysine-oligonucleotide probe complex, which is subsequently conjugated to polystyrene microparticle having a size around 2 micrometer to give the desired SAS. The chemistry for the coupling and conjugation is well known and can be easily found from references such as the tech notes from Bangs Laboratories.

The sample to be tested is mixed with 2 volumes of appropriate lysis buffer, e.g., 50 mM Tris-HCl, pH 7.4, 5 M guanidine thiocyanate and 2% Triton X-100, and rotated at room temperature for 20 minutes. Appropriate amounts of at least one pair of probes, which hybridize to different regions of the target RNA, are added to the lysed sample. The reaction mix is heated to 94°C for 5 minutes and then incubated at 50°C for 15 to 60 minutes to allow the probes to anneal to the target nucleic acids, which results in the formation of Probe A-Target RNA-Probe B complex. After addition of an appropriate amount of magnetic particles, e.g., 10^7 particles, the reaction mix is incubated for 15 to 60 minutes with agitation to allow the hybridization of all Probe A, which contains the hybridization domain for the polynucleotide probe on the magnetic particles. The reaction solution is then removed using a magnet. The magnetic particles are washed three times with 1 to 2 milliliters of washing buffer, e.g., phosphate saline buffer (PBS). If there is a sufficient amount of target nucleic acids in the sample, the magnetic particles will be labeled with Probe B through its binding to target nucleic acids.

To detect Probe B bound to the magnetic particles, the washed magnetic particles are suspended in 100 microliters hybridization buffer, e.g., PBS with 10 mM aminoethanethiol, 2% Tween 20. After addition of appropriate amounts of SAS, e.g., 10^6 particles, which is conjugated with an oligonucleotide that can hybridize with Probe B, the mix is incubated for 15 to 60 minutes under appropriate conditions that promote specific hybridization. The magnetic particles are then washed to remove unbound SAS. The bound SAS is detected through an appropriate instrument such as a luminometer. It may be necessary or preferred that chemiluminescent compounds on SAS are released from the particles before detection. Similarly, using magnetic particle as capture surface and Eu containing SAS particle can also give good result for fluorescent detection.

EXAMPLE 4

This example uses a rare element, chelated Europium (Eu), as an electrochemoluminescent agent for making SAS. Eu can be coupled to a carrier, e.g., dendrimer or linear polymer, through a chelator such as EDTA, which is coupled to the polymer using well known chemistry. The chelator-conjugated polymer can be used to prepare microparticles, so that the particles are encapsulated with chelators while particle surface is coated with functional groups such as carboxyl group. The chelator-containing microparticles can be used to trap Eu thereby creating Eu containing microparticles, which can now be labeled with affinity groups to create an SAS. The trapped Eu is preferably released from the particle prior to detection to improve detection efficiency.

Alternatively, the oxides of rare elements, e.g., Eu oxide, can also be directly coated with polymers with functional groups such as carboxyl group, which can

be used for affinity group labeling. The encapsulated Eu oxide powder are dissolved using an acid before measurement for its electrochemoluminescent activities.

The above methods use SAS only once in the detection, it is essentially a single round amplification method. With small modification to the above SAS and detection protocol, multiple rounds of amplification can be achieved by using two or more SAS sequentially. Normally only one of the SAS types contains the signal molecules, e.g., chemiluminescent compounds whereas the other SAS types contain multiple other SAS detectable markers instead of the signal molecules such as chemiluminescent or fluorescent molecules. The SAS detectable markers are molecules that can be detected by another SAS system; they could be single or double strand DNA, RNA, PNA (peptide nucleic acid) or the like, antibodies, antigens, small molecules, the affinity groups themselves or any chemical entities as long as they can be detected by another SAS system used in the next round of amplification. They could be either encapsulated inside or labeled on the surface if micro particles are used as carriers. Preferably, these markers are releasable. One example is given here: in order to detect antigen A, the first SAS contains multiple anti A antibody but not chemiluminescent group. The detectable marker in the first SAS is the affinity group itself: the antibody. Similarly to the procedure described in the above examples, after binding and washing, antibody in the first SAS is released. Because only a few copies of antigen A can give a lot more released antibody, the signal is amplified in the first round.

The second round of amplification involves the second SAS, which are coated with multiple aptamers that have affinity for the antibody resulted from the first SAS. The second SAS is added to the resultant reaction mix of the first amplification round. After binding and washing to remove unbound second SAS, the aptamers are released from the second SAS to achieve a second round of amplification. Similarly, the third round amplification uses the third SAS having multiple DNA sequence complementary to the released aptamers. Again after binding and washing, huge amount of DNA copies can be released from the third bounded SAS. If the amplification is great enough, the resulting DNA can be detected using traditional detection methods or another SAS system having chemiluminescent compounds or fluorescent compounds. Alternatively more rounds of amplification can be achieved using more of this kind of SAS before the final detection. In this example, the next SAS detectable markers are the affinity group. It can also be other molecules, for example, one SAS having antibody as affinity group and DNA or PNA as the next SAS detectable markers. The higher the SAS detectable markers one SAS unit can carry, the higher the amplification power one can achieve in each round. Preferably, one unit of SAS can carry more than one thousand copies of next SAS detectable markers.

EXAMPLE 5

This example illustrates that more than one cycle of amplification processes can be accomplished to achieve event greater signal amplification. This example is better understood by referring to Figure 7, which depicts two rounds of amplification processes using two SAS types and one magnetic capture bead type.

As shown in Figure 7, each SAS type is encapsulated with chemoluminescent compounds and coated with two affinity group types. The first SAS 11 is coated with an analyte-specific group 12 and another affinity group 13, e.g., biotin. The second (and last) SAS 14 is coated with only one affinity group 14, which is specific for affinity group 13 on the first SAS 11, e.g., avidin or its derivatives.

To conduct an assay, the analyte 5 to be detected is first immobilized onto a solid phase such as magnetic particles 15 shown in Figure 7. The magnetic particles are then incubated with first SAS 11 under conditions that promote specific binding between SAS 11 and magnetic particles 15 via the analyte. After removal of unbound SAS 11 through washing, the second SAS 14 is added and incubated again to allow the binding of SAS 14 with SAS 11 through the affinity groups, e.g., biotin-avidin binding. After removal of unbound SAS 14, the encapsulated chemoluminescent compounds in both SAS 11 and SAS 14 are released for detection.

It is evident that more than two rounds of amplification can be accomplished by simply designing additional SAS with appropriate affinity groups, which can be DNA (deoxynucleic acids), RNA (ribonucleic acids), PNA (peptide nucleic acids), or other ligand-receptor binding system.

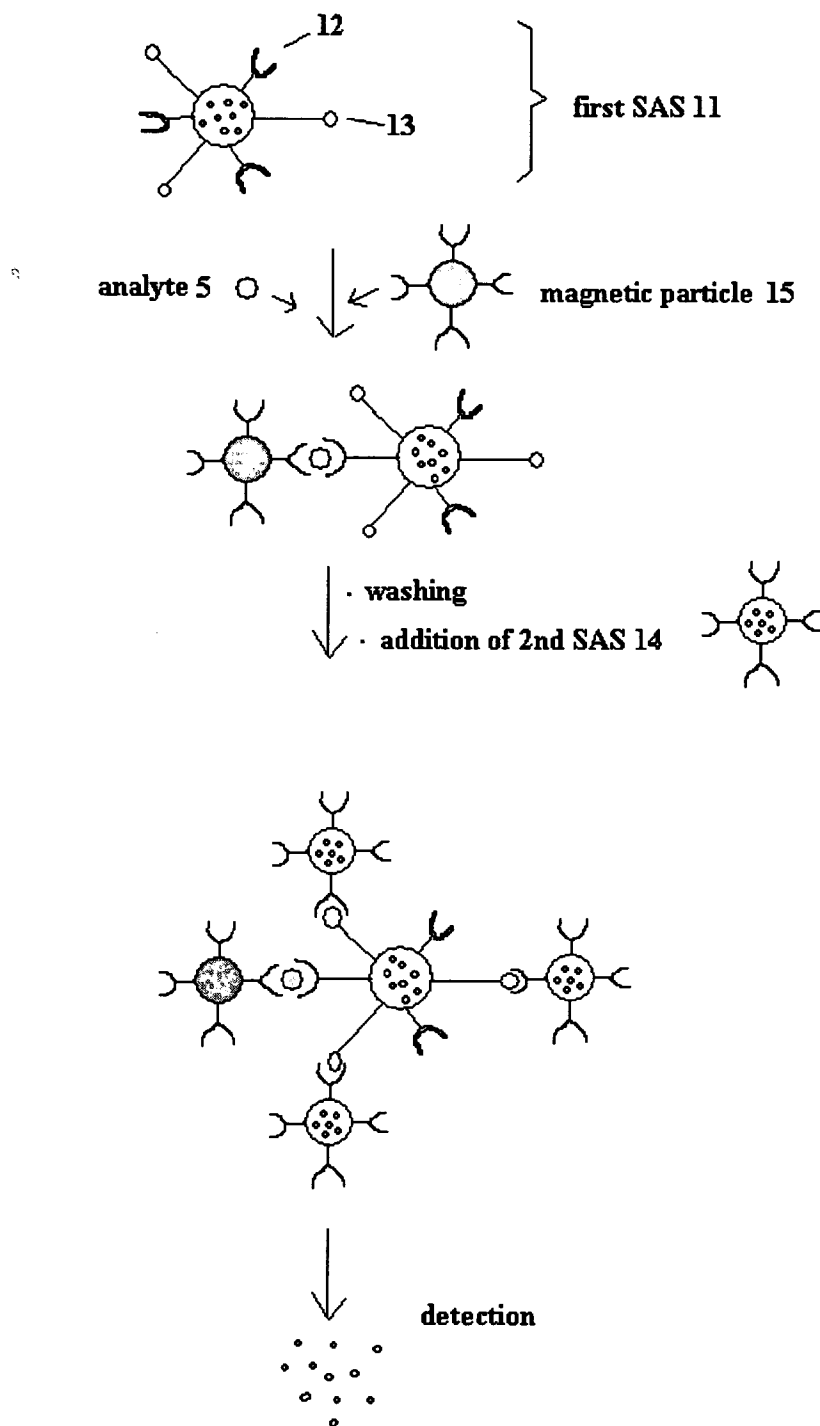


Fig 7. Two round amplification using one magnetic capture particle type

EXAMPLE 6

This example illustrates yet another example of multiple rounds of amplification processes. The difference is in the composition of the first SAS 16, which is coated or encapsulated with a larger number of linker molecule (next SAS detectable markers) that has two binding domains. The linker molecule can be DNA, RNA, PNA, ligand-receptor binding system, an antigen with at least two

epitopes, or a combination of the two. The more linker molecules can be coated onto SAS, the higher amplification power can be achieved. Preferably, more than 1,000 linker molecules are coated or encapsulated onto each SAS 16 unit.

In this case, as shown in Figure 8, the first SAS 16 is coated with a linker 19 (next SAS detectable markers) in addition to an affinity group specific for an analyte. This linker 19 is an oligonucleotide (oligo dA) coupled with biotin and therefore has two affinity domains: oligo dA and biotin. The oligo dA is preferably 30 base or longer. The linker is attached to SAS 16 through complementary hybridization with oligo dT, which is covalently conjugated to SAS 16. The second SAS 17 is similar to that in EXAMPLE 5 in that it is coated with an affinity group, e.g., avidin, for a binding domain of the linker, e.g., biotin. SAS 17 is coated or encapsulated with detection compounds, e.g., chemiluminescent compound.

To conduct an assay, the analyte 5 to be detected is first immobilized onto a solid phase such as first magnetic particles 18, which is coated with an affinity group for capturing the analyte (Figure 8). The magnetic particles 18 are then incubated with first SAS 16 under conditions that promote specific binding between SAS 16 and first magnetic particles 18 via the analyte. After removal of unbound SAS 16 through washing, the linker molecules are released from SAS 16.

The method for releasing the linker depends on how it couples to SAS 16. Appropriate coupling methods include, but are not limited to, photo-labile bond, reducing agent sensitive bond and nucleic acid hybridization. If the linker is coupled to SAS through a photo-labile bond, then the reaction mix can be subjected to irradiation of appropriate light. If the coupling bond is sensitive to reducing agent as in the case of disulfide bond, then the reaction mix can be subjected to reducing agent. In this example, the linker is coupled to SAS 16 through oligo dA:oligo dT hybridization. Therefore, the linker 19 can be released by adjusting the pH or, preferably, temperature. There are now a large number of released linker molecules in the reaction even if there are only a few specifically bound SAS 16.

The assay then proceeds to second amplification step, which uses the released linker molecule 19 in the reaction solution. A number of methods can be used to detect the released linkers. Described here is one example. Second magnetic particles 20 are added to the reaction mix preferably, but not necessarily, immediately before the linker release step. Magnetic particles 20 are coated with oligo dT, which is preferably 25 base or longer. The amount of added magnetic particles 20 should satisfy the desired upper and lower limit of detection or be in significant excess of the amounts the number of bound SAS 16 multiplied by the number of the linker on one SAS 16 unit, preferably with a ratio of at least 10 to 1 in unit numbers. The reaction mix is now heated to a temperature at which the linkers can be released from SAS 16. The temperature is then lowered to a temperature that permits hybridization between oligo dT and oligo dA in the linker. Because second magnetic particles 20 greatly outnumber SAS 16, most linker will hybridize with the second magnetic particles 20 even though SAS 16 is also coated with oligo dT. The second magnetic particles 20 are now coated with biotin, which is conjugated to the linker. It is not necessary, but may be preferred, to wash away SAS 16 before proceeding to the next step.

The second SAS 17, which is coated with avidin and signal molecules (e.g., chemiluminescent compounds), is then added to the reaction mix. After an incubation to allow avidin-biotin binding, the unbound SAS 17 is thoroughly washed away. The bound chemiluminescent compounds can be detected with an instrument such as a luminometer. The amounts of analyte 5 in the sample are proportion to the signal within linear range of the assay.

Evidently more than two rounds of amplification can be performed with the use of more linkers. It is within the scope of this invention that signal molecules may be something other than chemiluminescent and electro-chemiluminescent compounds. Appropriate signaling compounds or signal generating compounds include, but are not limited to, fluorescence generating compounds, color generating compounds, and enzymes that may generate such signals (e.g., peroxidase).

The amplification power primarily depends on how many linker molecules are incorporated into the first SAS 16 and how many signaling molecules are incorporated into the second SAS 17. For example, if 10,000 linker molecules are incorporated to the first SAS 16 and 10,000 signaling molecules are incorporated into the second SAS 17, then the amplification power would be 100 million with respect to the number of analyte molecules.

EXAMPLE 7

This example illustrates a preferred method of multiple rounds of amplification. This method is similar to that disclosed in EXAMPLE 6 except that the linker itself has an analyte binding domain, which can be a nucleic acids, antigen, antibody, ligand, receptor, aptamers (it can provide advantages of using nucleic acid pairing for the second round amplification), or any affinity entity that can provide specific binding for an analyte, or a combination of the two. The linker can be released from the first SAS 21 under conditions that preserve the binding capacity of the linker. Figure 9 depicts an assay using this amplification method.

To conduct an assay for the detection of analyte 5, the analyte molecules are first immobilized on a solid phase such as magnetic particles 22, which is coated with an affinity group specific for the analyte. The analyte-magnetic particle complex is then incubated with the first SAS 21, which is coated with the linker, which contains a binding domain specific for the analyte 5. The analyte binding groups on the first SAS 21 and first magnetic particles 22 are specific for different regions of the analyte 5. Magnetic particles 22 and SAS 21 can be added to the sample simultaneously, not necessarily sequentially.

After washing away unbound first SAS 21, the linker molecules are dissociated under a condition that preserves the binding capacity of the binding groups of the linker for the next round amplification. The second SAS 23 and second magnetic particles 24, which are coated with affinity groups for one or the other binding group on the linker, are added to the solution. Although in some cases the second magnetic particles 24 and first SAS 21 can compete for the same binding site on

the linker if the same binding site is used, most linker will bind to the second magnetic particles 24 since the second magnetic particles vastly outnumber the first SAS. The second SAS 23, linker and second magnetic particles form a complex in a sandwich format, which allows the removal of unbound second SAS 23 using, for example, a magnet. The signal molecules encapsulated in the second SAS 23 is released under appropriate conditions depending on the nature of the carrier and method of signal molecule conjugation/encapsulation, the signal is detected using appropriate instrument.

The number of captured second SAS 23 depends on the number of linker in the solution, which in turn depends how many linkers are coated onto the first SAS 21 and how abundant analyte 5 is. Because each first SAS 21 can be coated with numerous linkers, the signal is greatly amplified. For example, if 10,000 linker molecules are incorporated to the first SAS 21 and 10,000 signaling molecules are incorporated into the second SAS 23, then the amplification power would be 100 million with respect to the number of analyte molecules.

